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Optically Responsive Gold Nanorod-Polypeptide Assemblies

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Abstract

Environmentally responsive nanoassemblies based on polypeptides and nanoparticles can have a number of promising biological / biomedical applications. We report the generation of gold nanorod (GNR)-elastin-like polypeptide (ELP) nanoassemblies whose optical response can be manipulated based on exposure to near infrared (NIR) light. Cysteine-containing ELPs were self-assembled on gold nanorods mediated by gold-thiol bonds, leading to the generation of GNR-ELP nanoassemblies. Exposure of GNR-ELP assemblies to near-infrared (NIR) light resulted in the heating of gold nanorods due to surface plasmon resonance. Heat transfer from the gold nanorods resulted in an increase in temperature of the self-assembled ELP above its transition temperature (T_t), which led to a phase transition and aggregation of the GNR-ELP assemblies. This phase transition was detected using an optical readout (increase in optical density); no change in optical behavior was observed in case of either ELP alone or GNR alone. The optical response was reproducible and reversible across a number of cycles following exposure to or removal of the laser excitation. Our results indicate that polypeptides may be interfaced with gold nanorods resulting in optically responsive nanoassemblies for sensing and drug delivery applications.

Keywords

Photoresponsive; gold nanorods; elastin-like polypeptides; near infrared; surface plasmon; nanoassemblies

INTRODUCTION

Novel optically responsive nanoassemblies can have promising applications in molecular-scale switching devices,^{1, 2} sensors,³⁻⁵ drug delivery systems,⁶⁻¹⁰ and biomedical imaging modalities.^{11, 12} In particular, the interfacing of proteins / polypeptides with nanoparticles can result in the generation of novel functional nanomaterials for biological / biomedical applications. Gold nanorods demonstrate a tunable photothermal response to near infrared (NIR) light as a function of nanoparticle aspect ratio^{13, 14} and have been investigated as potential diagnostics,^{15, 16} therapeutic systems^{11, 17-19}, imaging agents,²⁰ and sensors²¹⁻²³. The ability to convert incident light energy to heat energy due to surface plasmon

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Conflict of Interest: None

Supporting Information Available. Figures showing absorption spectra of GNR-C2ELP (λ_{max} = 720 nm) nanoassemblies below and above the C2ELP transition temperature (T_t), and the experimental set up are included in the Supporting Information Section. This information is available free of charge via the Internet at <http://pubs.acs.org>.

resonance activity makes gold nanorods attractive candidates for modulating polypeptide (or protein) structure / phase transition using optical methods (i.e. near infrared light). While the ability to induce irreversible structural change in proteins can play a role in therapeutic applications, the ability to reversibly control protein structure lends flexibility for a variety of applications including site-specific drug delivery, biosensors, and switching.

Elastin-like polypeptides (ELPs) are derived from a portion of mammalian elastin characterized by the sequence, VPGXG, where V=valine, P=proline, G=glycine, and X=any amino acid except proline. ELPs exhibit a thermally induced phase transition at the inverse transition temperature, characterized by reversible intramolecular contraction and intermolecular coacervation²⁴. The thermal transition behavior of ELPs has been exploited in a number of applications including, bioseparations²⁵⁻²⁷, drug delivery²⁸⁻³², sensors³³⁻³⁵, and tissue engineering^{36, 37}. These reports have traditionally employed thermal activation as means of inducing phase transitions in ELPs. However, the ability to remotely and reversibly control the thermal response may be critical in applications such as targeted hyperthermia to tumors that may require precise spatial control in deep seated tissues.

We report gold nanorod (GNR)-elastin-like polypeptide (ELP) nanoassemblies that demonstrate a reversible optical response following exposure to near-infrared (NIR) light (Scheme 1). Energy from NIR light is converted to heat energy by gold nanorods which, in turn, is transferred to ELPs resulting in a reversible phase change in response to optical stimulation. Cysteine-containing ELPs were self-assembled on gold nanorods mediated by gold-thiol bonds, leading to the generation of GNR-ELP nanoassemblies. Exposure of GNR-ELP assemblies to near-infrared (NIR) light resulted in the heating of gold nanorods due to surface plasmon resonance.^{38, 39} Heat transfer from the laser irradiated gold nanorods resulted in an increase in temperature above the transition temperature (T_t) of the self-assembled ELP^{24, 25, 40} leading to aggregation of GNR-ELP assemblies. This was detected using an optical readout (increase in optical density); no change in optical behavior was observed in case of either ELP alone or GNR alone. The increase in absorbance was reversible following removal of the laser excitation.

EXPERIMENTAL

1. Design, Expression, and Purification of C₂ELP

C₂ELP Gene Synthesis—Cysteine-containing ELPs were synthesized via the method of recursive directional ligation,⁴⁰ yielding the following amino acid sequence, where cysteine residues are shown in bold and underlined font; the name C₂ELP denotes the two cysteines that are part of the ELP chain: MVSACRGPG-[VG VPGVG VPGVG VPGVG VPGVG VPG]₈-[VG VPGVG VPGVG VPGCG VPGVG VPG]₂-WP

Briefly, oligonucleotides encoding the ELP were cloned into pUC19, followed by cloning into a modified version of the pET25b+ expression vector at the *sfi*I site.⁴⁰ *E. coli* BLR(DE3) (Novagen) was used as a bacterial host.

C₂ELP Expression and Purification—The pET25b+ vector containing C₂ELP cassette was transformed into BLR cells. A starter culture of 50 ml was then inoculated overnight in terrific broth. The next day the 50 ml culture was added to a 1 liter culture. The 1 liter flasks were then inoculated overnight in an incubator shaker at 250 rpm and 37°C. Bacterial cells were harvested by centrifugation at 4°C the next day. The bacterial pellet was resuspended in 1X phosphate buffered saline (PBS) and the cells were disrupted by sonication on ice. The lysate was cleared by centrifugation followed by a polyethyleneimine treatment (final concentration: 0.5 % w/v) in order to precipitate soluble nucleic acids. After another round of centrifugation to pellet nucleic acids, the cleared supernatant containing C₂ELP was transferred

to a clean centrifuge tube. The tube was heated to 40°C in presence of 1M NaCl to precipitate the ELP. A warm centrifugation at 40°C was carried out to pellet C₂ELP. The supernatant was then discarded and the pellet was re-solubilized in PBS in the presence of 10 mM DTT. Another cold spin at 4 degrees was performed to get rid of insoluble contaminants. This cycle was repeated two more times. For the final resuspension step, the ELP was resuspended in purified water and then lyophilized and stored at room temperature.

C₂ELP characterization—The lyophilized C₂ELP was resuspended in 1X phosphate buffer saline (PBS). The resuspended material was then subjected to SDS-PAGE under denaturing conditions in order to determine the purity of the polypeptide. The gel was then stained with simply blue safe stain (Invitrogen, Carlsbad, CA) to visualize the proteins. As seen in Figure 1a, C₂ELP corresponds to a molecular weight of ~22 kDa.

2. Determination of T_t of C₂ELP

The transition temperature (T_t) of C₂ELP was characterized by monitoring the absorbance at 610 nm as a function of temperature with a UV-visible spectrophotometer (Beckman DU530) in 0.5X PBS. Briefly, 1 ml of C₂ELP was placed in a disposal cuvette. The temperature of C₂ELP was tuned by placing the C₂ELP-contained cuvette into a Precision 288 Digital Water Bath (Thermo Scientific) and was recalibrated by Digi-Sense Type J Thermocouple before absorbance measurement. The absorbance of C₂ELP was monitored at 610 nm with a UV-visible spectrophotometer (Beckman DU530) immediately after withdrawing the cuvette out of the water bath. The T_t is defined as the temperature at which the absorbance of C₂ELP solution reaches 50% of the maximum value. The temperature response of the C₂ELP indicated a T_t value of 33.4°C (Figure 1b). Note that we chose absorbance at 610 nm for determining the T_t value since gold nanorods show the lowest absorbance at this wavelength (Figure S1, Supporting Information Section) as a result of which, the absorbance of the solution is indicative of turbidity increase due to C₂ELP alone.

3. Generation of Near Infrared (NIR)-Absorbing Gold Nanorods

Gold nanorods were synthesized using the seed-mediated method as described by El-Sayed et. al.⁴¹ Briefly, the seed solution was prepared by adding iced water-cooled sodium borohydride (0.01M) to reduce a solution of 0.2 ml CTAB (cetyltrimethylammonium bromide) in 0.0005 M auric acid (HAuCl₄·3H₂O). The growth solution was prepared by reducing 0.2 ml CTAB in 0.001 M auric acid (HAuCl₄·3H₂O) containing 0.004 M silver nitrate with 0.0788 M L-ascorbic acid solution. Seed solution (12 µl) was introduced to 10 ml growth solution which resulted in the generation of gold nanorods after 4 hours of continuous stirring. This method was employed for generating two different gold nanorods that possessed absorbance maxima at (λ_{max}) at 710 nm and 810 nm, respectively. Each nanorod population was diluted with DI water to an optical density ~0.5 (Figure 1c).

4. Generation of Gold Nanorod / C₂ELP Nanoassemblies

C₂ELP (2mg/mL in 1X PBS) was added into DI water at a 1:1 volume ratio, to form C₂ELP solution (1mg/mL in 0.5X PBS; “C₂ELP alone”). Gold nanorods (1ml; λ_{max}=710 nm or 810 nm) in DI water having an absorbance of ~0.5 were added to an equal volume (1 mL) of 1X PBS in order to bring the final concentration to 0.5X PBS (“GNR alone”). The absorbance of the resulting solution was verified to be similar for all cases following mixing. C₂ELP (T_t = 33.4°C) was self-assembled on gold nanorods overnight leading to formation of the nanoassemblies via gold-thiol bonds (“GNR- C₂ELP assemblies”). Briefly, 1ml of C₂ELP (2mg/mL in 1X PBS) was mixed with 1 ml of GNR solution in DI Water to form a 2 ml GNR-C₂ELP solution (1mg/mL in 0.5X PBS). Prior to self-assembly, 20 milligrams of Reductacryl® resin (EMD Biosciences Inc.) were added to C₂ELP (1ml) for 15 min in order to reduce the

cysteines in the polypeptide. Reduced C₂ELP was separated from the Reductacryl® resin by centrifugation at 13,000 rpm for 10 minutes and immediately added to gold nanorods at a volumetric ratio of 1:1 and stirred overnight at room temperature. Equivalent concentrations of gold nanorods (without self-assembled C₂ELP) and C₂ELP (without gold nanorods) were used as controls in the subsequent experiments.

5. Optical Response of GNR-C₂ELP Nanoassemblies

A titanium CW sapphire (Ti:S) laser (Spectra-Physics, Tsunami) pumped by a solid state laser (Spectra-Physics, Millennia) was used for the laser irradiation experiment. The excitation source was tuned to 720 nm or 810 nm in order to coincide with the longitudinal absorption maximum of the GNR-C₂ELP nanoassemblies and GNRs in the two cases, respectively. In case of GNR ($\lambda_{\text{max}} = 710$ nm), self assembly with C₂ELP resulted in a slight red shift of the absorbance peak to 720 nm resulting in GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) (Figure 1d). As a result, the laser was tuned to the absorption maximum of the nanoassemblies. In the second case GNR ($\lambda_{\text{max}} = 810$ nm), we used a laser wavelength that corresponded with the absorption maximum of the nanorods and not the nanoassemblies based on the observation with GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) that indicated that self-assembly of the C₂ELP did not result in a significant shift of the absorption spectrum.

As with the T_t measurements before, we employed absorbance at 610 nm for evaluating the optical response of the GNR-C₂ELP nanoassemblies. The absorption spectrum of the nanoassemblies below (25°C) and above (35°C) the T_t (33.4°C) is shown in Figure S1; this spectrum was determined using temperature controlled absorbance spectroscopy in a plate reader (Biotek Synergy 2) containing the nanoassemblies (i.e. no laser irradiation was employed for determining the spectrum). A comparison of the spectra below and above the transition temperature indicates that while the nanoassemblies did not demonstrate high absorbance values below the T_t , an increase in solution turbidity resulted in high absorbance values above the T_t .

The optical response of 1 ml GNR-C₂ELP assemblies in response to laser exposure in 0.5X PBS was first characterized by monitoring the absorbance at 610 nm using with a UV-visible spectrophotometer (Beckman DU530) as a function of the employed laser power. Briefly, GNR, C₂ELP, and GNR-C₂ELP solutions (1 ml in disposal cuvettes) were exposed to laser light at different power densities (0 mW – 510 mW) for 5 minutes. The laser, tuned to 720 nm or 810 nm (3 mm or 1mm diameter beam, respectively) was focused through the center of the cuvette in a vertical fashion (Figures S2). The temperature of the three solutions was monitored as a function of the laser power using a Digi-Sense Type J Thermocouple.

In order to obtain the kinetics of the optical response and the corresponding temperature response, cuvettes containing 1 ml of GNR-C₂ELP solution, GNR solution or C₂ELP solution were exposed to a maximum laser power (460mW for GNR ($\lambda_{\text{max}} = 720$); 510mW for GNR ($\lambda_{\text{max}} = 810$) for different periods of time (0min - 10min) beginning at room temperature. The optical response was characterized by monitoring the absorbance at 610 nm as a function of time with a UV-visible spectrophotometer (Beckman DU530); as before, the kinetics of the temperature response was monitored using a Digi-Sense Type J Thermocouple.

RESULTS AND DISCUSSION

Environmentally responsive biomolecules and nanoparticle-biomolecule systems are being actively pursued with an eye towards enhancing sensing, therapeutic, and device capabilities⁴². Temperature-based control is a powerful strategy for generating robust environmentally responsive systems. However, localized control of temperature can be of importance in biological applications including tumor-targeted hyperthermia,⁴³ functional

devices,⁴⁴ and cell patterning⁴⁵. In the present study, we have interfaced the temperature-responsive properties of elastin-like polypeptides (ELPs) with the ability to remotely tune the thermal response of gold nanorods using near infrared irradiation in order to generate optically responsive nanoparticle-polypeptide assemblies.

A newly designed 22 kilo Dalton (kDa), cysteine-containing elastin-like polypeptide (C₂ELP; Figure 1a), was cloned using recursive directional ligation, expressed in *E. Coli* and purified as described previously.⁴⁰ The transition temperature (T_t) of C₂ELP was experimentally determined to be 33.4°C (Figure 1b). Gold nanorods (GNRs), with peak absorbance in the near infrared region of the absorption spectrum (710 nm and 820 nm; Figure 1c) were generated using the seed-mediated chemical synthesis method.⁴¹ C₂ELP ($T_t = 33.4^\circ\text{C}$) was first reduced using Reductacryl® (in order to reduce cysteines) and then self-assembled on GNR ($\lambda_{\text{max}} = 710\text{ nm}$), i.e. gold nanorods whose peak absorbance (λ_{max}) was at 710 nm. Upon incubation with C₂ELP, the maximal absorbance peak showed a slight red-shift to 720 nm due to the self assembly of the polypeptide on the nanorods⁴⁶⁻⁴⁸ (Figure 1d) indicating the formation of GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) nanoassemblies.

Next, we determined the optical response of the GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) nanoassemblies using near infrared (NIR) laser-irradiation. Figure 2a shows the change in absorbance as a function of laser power following exposure of the GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) nanoassemblies to a 720 nm NIR laser for 5 minutes. While no change in solution optical density was observed for laser powers below 300 mW, a sharp transition and strong optical response were observed for laser powers in excess of 350 mW, indicating that energy from the laser was converted into heat due to the surface plasmon property of gold nanorods. This, in turn, resulted in an increase in temperature of the self-assembled elastin, leading to the aggregation of GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) and the observed optical response. We measured the solution temperature using a J-thermocouple (Figure 2b) and correlated it with the profile with laser power employed in order to investigate the observed photothermal response. The observed temperature at a laser power of 300 mW was 32.8°C, which is below the T_t (33.4°C) of the self-assembled C₂ELP. However, when the laser power was increased to 400 mW, the temperature of the GNR-C₂ELP solution reached 35.3°C ($> T_t$) following the five minute NIR exposure. This resulted in a sharp increase in the optical density, indicating that laser powers higher than 350 mW were required for inducing the optical response. The range of laser power (350-650 mW) and / or power densities (5-7 W/cm²) that induce the optical response described in the above experiments is either significantly lower^{47, 49} or comparable^{50, 51} to those used in other reports in the literature on NIR-mediated photothermal activation of gold nanoparticle-based systems. These results are consistent with expected behavior in that, the optical response due to ELP self-assembly was seen beyond a threshold laser power which was necessary to raise the ELP temperature beyond its corresponding T_t .

The kinetics of the optical response were then investigated by measuring the absorbance of GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) as a function of time (Figure 2c); in addition the kinetics of the optical response were correlated with the kinetics of temperature increase (Figure 2d) in order to explain the photothermal effect. Figure 2c shows the optical response of GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$), GNR, and C₂ELP as a function of laser exposure time. The laser power was fixed at the maximum power (460 mW) used in the previous experiments (Figure 2a) in order to reliably generate the maximal optical response. The optical density of the GNR-C₂ELP ($\lambda_{\text{max}} = 720\text{ nm}$) nanoassemblies increased sharply after two minutes of laser exposure and reached a plateau after five minutes. Although the maximal optical response was observed five minutes following laser exposure, a detectable response was observed after only two-and-a-half minutes of laser exposure time. In contrast, no change in optical density was seen either in the case of nanorods alone or C₂ELP alone, indicating that the optical response was specific to nanorod-polypeptide assemblies. Figure 3 shows digital snapshots of the time-dependent

phase transition and optical response of GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) nanoassemblies as a function of time following laser exposure (laser power = 460 mW); the respective laser exposure times are shown by the timer in the background. As seen in the figure, the GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) solution continued to be optically clear after one minute of laser exposure time. However, the solution turbidity increased after three minutes of laser exposure and reached a maximum in five minutes. The optical density remained invariant following five minutes of laser exposure consistent with the absorbance measurements in Figure 2c.

The temperature response of the GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) was determined as a function of time and compared with the response of solutions containing C₂ELP alone and nanorods alone. The observed temperatures of the GNR-C₂ELP solution were 33.6 °C, 35.5 °C, and 37.4 °C following 3, 4, and 5 minutes of exposure with 720 nm laser, respectively (Figure 2d). The time required for the solution temperature to increase beyond the transition temperature of C₂ELP following NIR irradiation was well correlated with the observed change in solution optical density (Figure 2c). In addition, the kinetics of temperature increase of GNR-C₂ELP nanoassemblies closely followed the rise in temperature of GNR alone. Thus, although the temperature of GNRs alone rose faster than GNR-C₂ELP nanoassemblies (as may be expected), no change in optical response was observed in the former case (Figures 2c and 2d). No change in solution temperature was observed with C₂ELP alone upon exposure to the laser, which explains the lack of optical response in this case.

In order to examine if the observed optical response was reversible, GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) assemblies were subjected to five alternating cycles in which the laser exposure was turned on for 5 minutes and then turned off for 5 minutes. Following laser exposure (laser power = 460 mW) for five minutes, the absorbance of the solution increased up to 1.2 absorbance units (AU) indicating formation of the aggregated nanoassemblies (Figure 4). The optical density of the solution returned to baseline values in approximately two minutes following removal of the laser excitation. This behavior was reproducible over the five cycles tested, indicating that the optical response of the GNR-C₂ELP assemblies was indeed reversible and reproducible across multiple cycles.

The optical response was also evaluated with another set of gold nanorods that possessed peak absorbance at 810 nm. In the case of these nanorods, i.e. GNR ($\lambda_{\text{max}} = 810$ nm), the laser wavelength was tuned to the maximal peak absorbance of the nanorods (i.e. 810 nm) and not to the peak absorbance of the nanoassemblies. ELP self-assembly was shown to result only in a minor red-shift of the peak absorbance peak (Figure 1d) of the nanorods. As a result, the laser was tuned to the peak absorbance of the nanorods in this case. As with the GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) assemblies, the temperature profile (Figure 5b) of these nanoassemblies closely followed the laser power employed (Figure 5a). The optical response was specific to the GNR-C₂ELP nanoassemblies; no change in absorbance was seen with solutions containing C₂ELP alone and nanorods alone (Figure 5c). Finally, the optical response of the GNR-C₂ELP nanoassemblies closely followed the kinetics of temperature increase (Figure 5d). The increase in temperature in case of GNR ($\lambda_{\text{max}} = 810$ nm)-C₂ELP closely followed that of GNR ($\lambda_{\text{max}} = 810$ nm) alone. However, the optical response was seen only in case of the former due to the presence of the self-assembled C₂ELP. These results are consistent with those observed with the GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) assemblies indicating that the optical response can be obtained with nanorods that absorb at different wavelengths of the near-infrared region of the absorption spectrum, which further expands the application range of these nanoassemblies.

CONCLUSIONS

We have investigated novel optically responsive polypeptide-based nanoassemblies in which, heat transfer from NIR-absorbing gold nanorods resulted in a conformational change in the

self-assembled elastin-like polypeptide (ELP) leading to a detectable optical response. Such ability to control nanoscale assembly and nanomaterial properties by optical manipulation can be exploited in the development of novel sensors, drug delivery systems, functional molecular and nanoscale devices, and imaging agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.
Schematic of optically responsive gold nanorod (GNR)-elastin-like polypeptide (ELP) nanoassemblies.

Figure 1a. Denaturing SDS-PAGE analysis of C₂ELP employed in the current study. The molecular weight of the polypeptide was 22kDa.

Figure 1b.

Temperature-dependent phase transition of C₂ELP as measured by change in optical density at 610 nm indicates a transition temperature (T_p) of 33.4°C.

Figure 1c.

Absorption spectra of gold nanorods generated using the seed-mediated method⁴¹. Two separate populations of gold nanorods were generated as described in the experimental section and their absorbance was determined as a function of wavelength; the nanorods demonstrated peak absorbances at 710 nm and 810 nm and are called GNR (710 nm) and GNR (810 nm), respectively.

Figure 1d.

Absorbance spectrum of GNR ($\lambda_{\text{max}} = 710$ nm) and GNR ($\lambda_{\text{max}} = 710$ nm)-C₂ELP nanoassemblies at 25°C ($< T_f$). A red shift can be observed in the longitudinal peak in case of the nanorod polypeptide assemblies resulting in peak absorbance at 720 nm of the GNR-C₂ELP ($\lambda_{\text{max}} = 720$ nm) nanoassemblies.

Figure 2.

Optical response (in absorbance units or AU at 610 nm) of gold nanorod (GNR; $\lambda_{\max}=720$ nm)-C₂ELP assemblies. (a) Optical response and (b) temperature response as a function of laser power following 5 minute exposure to 720 nm laser. Kinetics of (c) optical and (d) temperature response of GNR-C₂ELP assemblies following excitation with 720 nm laser. Lines connecting data points are for visualization only.

Figure 3.

Digital images of the optical response of GNR ($\lambda_{\text{max}} = 720 \text{ nm}$)-C₂ELP nanoassemblies as a function of laser exposure time. Change in optical density remained invariant after 5 minutes following laser exposure. The corresponding laser exposure times can be seen in the timer in the background.

Figure 4.

Reversibility of optical response of GNR-C₂ELP ($\lambda_{\text{max}} = 720 \text{ nm}$) assemblies. GNR-C₂ELP assemblies were subjected to alternating cycles laser exposure in which the laser was turned on and off for five minutes each. The absorbance of the suspension was noted as a function of time.

Figure 5.

Optical response (in absorbance units at 610nm) of gold nanorod (GNR; $\lambda_{\max}=810\text{ nm}$)-C₂ELP assemblies. (a) Optical response and (b) temperature response as a function of laser power following 5 minute exposure to 810 nm laser. Kinetics of (c) optical and (d) temperature response of GNR-C₂ELP assemblies following excitation with 810 nm laser. Lines connecting the data points are for visualization only.